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The inflammation-induced pathological chaperones ACT and apo-E are necessary catalysts of Alzheimer amyloid formation

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Abstract

Biochemical, genetic, and epidemiological evidence indicates that inflammation is an essential part of the pathogenesis of Alzheimer's disease. Over the last decade, we and others have focused on the mechanism by which specific inflammatory molecules contribute to the Alzheimer pathogenic pathway. In particular, we have learned that several acute phase/inflammatory molecules, specifically α_1-antichymotrypsin (ACT) and apolipoprotein E (apoE) that are overproduced in the AD brain can promote the formation of, and are associated with, the neurotoxic amyloid deposits that are a key pathological hallmark of the disease. Because both of these proteins bind to the Aβ peptide and catalyze its polymerization into amyloid filaments, they have been termed "pathological chaperones".

ACT, and, to a lesser extent, apoE are greatly overproduced only in areas of the AD brain that are prone to amyloid formation. This restriction suggests a local inflammatory reaction may underlie the regional specificity of amyloid deposition by inducing the production of pathological chaperones. The data that will be discussed indicate that ACT over-expression is caused by the activation of ACT mRNA synthesis in astrocytes in response to increased production of the inflammatory cytokine IL-1. IL-1 is released from microglia that become activated by pre-amyloid seeds of Aβ. Recently, this inflammatory cascade has been extended to include the amyloid precursor protein (APP), for IL-1 also upregulates the production of APP in astrocytes, but at the translational rather that the transcriptional level. Thus many of the key elements of the Alzheimer's disease pathogenic pathway are products of a local inflammatory reaction in the brain.

Further support for a mechanistic role of inflammation in the Alzheimer's disease pathogenic pathway has been provided by genetic studies, which have associated an increased risk of developing AD with specific polymorphisms in the apoE, ACT, and the IL-1 genes. Most recently, transgenic mouse models of AD have demonstrated that ACT and apoE are amyloid promoters/pathological chaperones *in vivo* whose contribution is necessary for both amyloid formation and for amyloid-associated cognitive decline and memory loss.

The importance of these findings is that they help to place inflammation at the center of the pathogenic pathway to Alzheimer's disease and identify specific steps in the pathway that may be amenable to therapeutic intervention.

1. Introduction

Alzheimer himself hinted at the possibility that Alzheimer's disease pathology might involve an inflammatory reaction when he described reactive astrocytes and microglia in affected brain regions of his first patient [4]. However, the absence of standard features of inflammation such as swelling and lymphocyte infiltration argued against such a description. A change of view began to arise in the 1980's when activated microglia in Alzheimer's disease brain were found to express HLA antigens characteristic of inflammation [67] and when the Alzheimer amyloid deposits were found to contain, in addition to Aβ peptides, other proteins that are normally secreted during inflammation and its associated acute phase response (for reviews, see [15,53]. For example, the inflammation/acute phase protein α_1-antichymotrypsin (ACT) was found to be a structural component of the Alzheimer amyloid deposits, but not to be associated with the deposits in other amyloidoses [1,3]. Furthermore, ACT mRNA and protein are undetectable in normal brain and are massively expressed in astrocytes in those parts of the AD brain, such as the hippocampus, that develop large numbers of amyloid plaques [1,33,56]. ACT is an inhibitor of chymotrypsin-like serine proteases and is normally pro-

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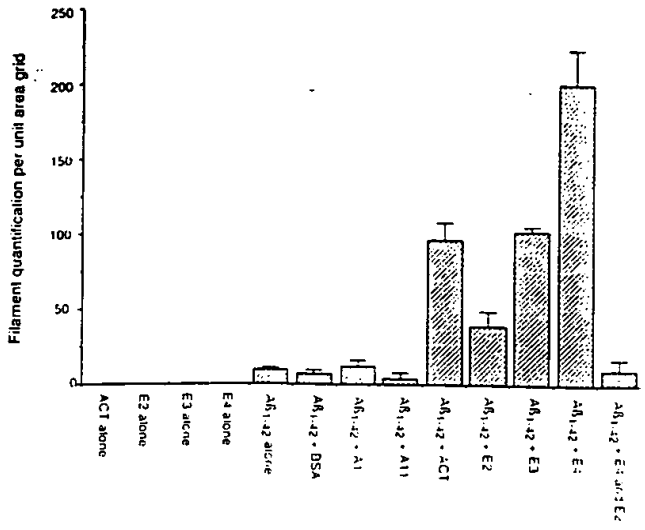

Fig. 1. Quantitative electron microscopic analysis of filaments of Aβ peptide formed either alone or in the presence of 1/200 molar equivalent of the indicated protein indicate that ACT and apoE4 are amyloid promoters and that apoE2 is an amyloid inhibitor *in vitro*. In contrast, the other proteins tested, BSA, apolipoprotein A1, and apolipoprotein A11 are not amyloid promoters. (from [41]; copyright 2000 Nature Publishing).

duced in the liver as part of the body's "acute phase response" to inflammation [77]. The finding that ACT is overexpressed in astrocytes in affected areas of the Alzheimer brain provided the first clear indication that inflammation and an acute phase response *in the brain* were part of the disease. The question that remained was whether inflammation was merely a response to the disease, or whether it was an essential cause.

In this paper, we will present both established and new evidence that implicates inflammation and the molecules it produces, particularly ACT, apoE, and IL-1, in Alzheimer amyloid formation. The conclusion is that Aβ does not function alone to cause Alzheimer's disease, but must interact with one or more pathological chaperones that serve to catalyze the polymerization of Aβ into amyloid filaments. In the absence of inflammation and amyloid promoters, Aβ alone might be relatively harmless.

2. The role of ACT and apoE in Alzheimer amyloid formation

The findings that ACT binds directly to the Aβ peptide and is an integral component of the Alzheimer amyloid filaments, and that the mature amyloid deposits are restricted to the same brain regions in which ACT is overproduced, led us to propose that ACT contributes directly to amyloid formation [1,59,62]. When ACT is added to preparations of synthetic Aβ peptide *in vitro*, it promotes the polymerization of Aβ into amyloid filaments ([41]; Fig. 1) which are toxic to neurons in culture [42]. Furthermore, biophysical experiments and molecular modeling have suggested that the Aβ peptide assumes a β-sheet conformation while inserting between two β-strands in the ACT protein [29,30,40]. By forcing a single Aβ molecule into a stable β-sheet, ACT can evidently initiate/catalyze the β-sheet-based self-assembly process needed for Aβ to polymerize into an amyloid filament. As will be discussed, recent genetic and transgenic mouse results support the hypothesis that ACT is an amyloid promoter in Alzheimer's disease, working at an early step in filament formation.

When apolipoprotein E was found to be also present in Alzheimer amyloid, suggesting a similar role to ACT, the term "pathological chaperone" was coined to describe the potential function of these two, and possibly other proteins, in amyloid formation [81]. It was then soon discovered that, besides age itself, inheritance of the apoE4 allele is the strongest known risk factor for developing Alzheimer's disease [11,57,74]. It was therefore striking that the apoE4 protein is a much more active amyloid promoting factor *in vitro* than the non-pathogenic apoE3 or apoE2 isoforms ([41,70,82]; Fig. 1). Furthermore, the greater number and length of the filaments formed under the promoting effect of both ACT and apoE4 showed increased toxicity to human cortical neurons in culture [42]. Finally, apoE2, which shows protective activity against Alzheimer's disease in epidemiological studies [12], also suppressed the ability of apoE4 to promote Aβ polymerization *in vitro* ([41]; Fig. 1). These results together with the finding that apoE4 individuals with Alzheimer's disease evidence greater amyloid load than do patients with only the common apoE3 allele [64,71] suggested that the mechanism by which apoE4 exerts its promoting effect on the development of Alzheimer's disease is through the promotion of Aβ polymerization and amyloid formation. Furthermore, the results suggested that, in general, amyloid promoters expressed during inflammation may be an important part of the disease process.

3. Are apoE and ACT amyloid promoters or amyloid inhibitors?

All of the pathological evidence (such as the overexpression of ACT and apoE in affected areas of AD brain and the increased amyloid load in apoE4 and ACT-A carriers) together with most of the biochemical evidence, has pointed to these proteins being amyloid promoters. However, the genetic data alone could not exclude the possibility that, for instance, apoE is an amyloid inhibitor with apoE4 being a less effective inhibitor than apoE3. Indeed, following our and other lab's reports that apoE and ACT are amyloid promoters, several investigators presented *in vitro* studies in which apoE or ACT appeared to inhibit Aβ polymerization (see for example [17,18,20,79]). We argued that the *in vivo* data could be more simply explained by a promotion model and that many of the *in vitro* experiments showing amyloid inhibition by ACT or apoE were carried out with the Aβ1-40 instead of the probably pathogenic Aβ1-42 [14,28].

Also, some experiments were carried out at near stoichiometric concentrations of the reagents, assuring that the Aβ-binding proteins would sequester all of the free Aβ and prevent polymerization. Such a result clearly reflected the proteins' interaction but was not necessarily relevant to the catalysis of the polymerization reaction. Indeed, an experiment performed by Janciauskiene et al. [30] clearly showed that ACT functions as an amyloid promoter at concentrations far below equi-molarity with Aβ and loses its promoting effect at higher concentrations. Nonetheless *in vitro* work by Webster and Rogers [79] with ratios of 5:1 and 20:1 between Aβ and an added protein showed no effect of ACT and an inhibitory effect of apoE on Aβ1-42 aggregation. Clearly *in vitro* experiments alone are insufficient to determine precisely how ACT or apoE affect amyloid formation in AD, and additional lines of investigation have been necessary to help clarify the role of these proteins in the disease process.

4. The involvement of IL-1 in the AD pathogenic pathway

Soon after the discovery of ACT in the amyloid deposits, another important piece of biochemical evidence suggesting the presence of inflammation in AD was provided by Griffin et al. [23,72]. They showed that the activated microglia in affected areas of the AD brain express large amounts of the inflammatory cytokine IL-1. This discovery was particularly interesting to us because IL-1 is the cytokine that upregulates ACT expression in hepatocytes as part of the body's acute phase response to inflammation [8]. When IL-1 is added to astrocytes, the cytokine is recognized by its receptor and induces a massive increase in transcription of the ACT gene ([13]; see also [34,43]). Furthermore, the areas of the human Alzheimer brain that the highest levels of expression of IL-1 in microglia are the same regions of human fetal brain that contain an apparently-special class of microglia that are capable of expressing IL-1. These results, together with the evidence that ACT is only expressed in those brain regions in Alzheimer's disease that express IL-1 and can promote neurotoxic amyloid formation *in vitro* [1,41,42] provides strong circumstantial evidence that an IL-1/ACT inflammatory cascade may contribute importantly to the pathogenesis of AD.

One of the main mysteries about Alzheimer's disease is that the major amyloid component—the Aβ peptide—is expressed throughout the body and the brain in both normal and Alzheimer individuals, and yet it deposits as mature amyloid only in specific regions of the Alzheimer brain. The identification of ACT and apoE4 as amyloid-associated and amyloid-promoting proteins, and IL-1 as a key inflammatory cytokine in AD brain provides a mechanism for the region-specific and disease-specific deposition of amyloid. All of these proteins are overexpressed only in Alzheimer's brain areas showing neuropathology. This suggests that the regional restriction of Alzheimer amyloid neuropathology might, in part, be due to the region-specific inflammatory cascade that leads to expression of amyloid promoting factors such as ACT and apoE as an important step in the Alzheimer pathogenic pathway [13,51,83].

Together, these results support the hypothesis that Alzheimer's disease involves an inflammation-like reaction and a consequent acute phase response in the brain that is essential for the development of mature amyloid neuropathology and neuronal cell death. This inflammatory cascade has been recently extended to include the amyloid precursor protein (APP) itself, by the finding that IL-1 upregulates the production of APP in astrocytes [68]. In contrast to ACT, APP upregulation by IL-1 is controlled at the translational rather than the transcriptional level.

5. Epidemiological studies

The finding that ACT and apoE are overexpressed in astrocytes in areas of Alzheimer brain showing pathology has also led to examinations of ACT levels in serum and cerebral spinal fluid (CSF) (for example: [37,38]; for review, see [53]). The majority of studies find ACT to be significantly elevated in the serum and CSF of AD patients, thus confirming the presence of inflammation that can be detected before death.

In addition to the biochemical and pathological evidence that inflammation plays a role in Alzheimer's disease pathogenesis, retrospective and, more recently, prospective studies on certain populations strongly support such a conclusion. For example, patients suffering from inflammatory diseases such as rheumatoid arthritis appear to have a reduced incidence of Alzheimer's disease [5,31,44]. The explanation for these results, as suggested by the authors, was that the non-steroidal anti-inflammatory drugs (NSAIDs) routinely used by these patients had protected against developing Alzheimer's disease. These early studies have been reproduced in larger experimental settings [5,45,65] as well as by alternative methods such as co-twin control studies [9]. Indeed, initial clinical trials demonstrated that the inflammatory drug indomethacin exerted beneficial effects on Alzheimer patients with respect to cognitive decline [66], although later studies failed to confirm a therapeutic effect of NSAIDs in already-diagnosed AD [69]. Interestingly, NSAIDs have been shown to inhibit the stimulatory effect of IL-1 on astrocytoma cells ([19]; Rogers and Potter, unpublished), suggesting that NSAIDs might need to be administered very early in the disease process in order to inhibit the early amyloid-promoting stages of the inflammatory cascade in AD.

6. Genetic support for the involvement of apoE, ACT, and IL-1 in Alzheimer's disease

Since the finding that ACT and apoE, especially apoE4, are amyloid promoters *in vitro*, many other proteins have

been tested and some have been found to affect Aβ polymerization (for review see [53]). However, thus far, ACT and apoE are the only potential pathological chaperones for which genetic studies also support their involvement in the Alzheimer pathogenic pathway. For example, as discussed above, one of the greatest genetic risk factors for developing Alzheimer's disease is the inheritance of one, or worse, two copies of the apoE4 allele. This epidemiological finding first made by Strittmatter, Roses and Poirier and their colleagues has been confirmed and extended in many subsequent studies [11,57,73,74]. Furthermore, several promoter mutations in the apoE gene that increase production of the protein have also been shown to confer increased risk of developing AD, further supporting the principle that apoE promotes Alzheimer amyloid formation [6,10,35,36].

Genetic support for the involvement of ACT in Alzheimer's disease is not as striking as for apoE4, but is nonethe-less becoming clearer. In the first study, the inheritance of a specific isoform of ACT (an alanine instead of a threonine in the signal peptide) correlated with a further 8-fold increased risk of developing Alzheimer's disease in apoE4 carriers [32]. Because the single-amino acid change caused by ACT-A does not affect the secreted protein itself and therefore cannot alter its affinity for the Aβ peptide or its ability to promote Aβ polymerization, Kamboh and colleagues proposed that it most likely effects the synthesis and secretion of the ACT protein.

Since the original Kamboh et al. paper was published [32], the potential risk factor status of the ACT-A allele has become controversial. Although the genetic risk of the ACT-A allele for the development of AD was confirmed and extended by some studies (see for example, [16,47,50, 75,76]) it was not confirmed by others (for example, [24, 27,49]). Recently, a particularly well-controlled and carefully-analyzed study in a Japanese population showed that inheritance of the ACT-A allele (independent of apoE alleles) was highly correlated with the extent of amyloid angiopathy in the brain, indicating that ACT directly influences amyloid formation in Alzheimer's disease [84]. There are several possible explanations for the inconsistency of the cited results. First the effect of the ACT-A allele might be small (as is suggested by experiments examining the effect of ACT-A on ACT secretion from transfected cells in culture; [54]; discussed below). A similarly small effect on AD risk might be masked in highly heterogeneous human populations. Another point is that some of the "negative" reports might well have shown an effect of ACT-A if the proper statistical tests had been performed. Other papers failed to perform a power calculation to show that their lack of association was significant, and thus would be better termed 'NON-results' rather than 'negative results'. Finally, it has been shown recently that the effect of ACT-A may depend on the presence of an additional presenilin polymorphic allele on the same chromosome 14, again potentially explaining the different results that have been reported [78].

We have obtained biochemical evidence in favor of the hypothesis that ACT-A promotes Alzheimer's disease [54]. Plasmids were constructed that would express either ACT-A or ACT-T after transfection into recipient cells. After transfection of the two plasmids into COS cells, the ACT-A expression is more efficient than the ACT-T expression as evidenced by the speed with which the two nascent proteins traverse the endoplasmic reticulum and become glycosylated in the Golgi. This result not only confirms Kamboh's and colleagues' prediction, but it is also consistent with the documented effect of replacing a relatively hydrophobic amino acid, such as alanine, with a more hydrophilic amino acid, such as threonine, in the signal peptide of a secreted protein. This ability of the ACT-A allele to increase the secretion of ACT, compared to the ACT-T allele, provides a reasonable mechanistic explanation for the enhanced risk for AD that many groups have found to be conferred by ACT-A. If ACT promotes amyloid filament formation, it is reasonable to assume that a genetic tendency to greater ACT production might promote greater polymerization of Aβ into amyloid filaments in individuals carrying the ACT-A allele.

The most recent genetic evidence that inflammation plays an important role in AD comes from the finding that polymorphic allele variants of the IL-1 promoter that increase IL-1 production confer as much as a 10-fold increased risk of developing AD [39,52]. This result is particularly interesting because of IL-1's apparent role in inducing the overexpression of the amyloid promoter ACT, of APP and Aβ, and, possibly indirectly, of apoE in affected areas of AD brain.

7. Alzheimer amyloid formation *in vivo*—proving the chaperone hypothesis with transgenic mice

The role of apoE in amyloid formation has recently been clarified by a series of *in vivo* experiments that confirm it to be an amyloid promoter [7,26]. First, a set of mouse strains were developed that expressed transgenic human APP but which had their apoE gene either half (heterozygous) or completely (homozygous) knocked out. The animals showed a variable amount and speed of amyloid deposition that was dependent on the number of copies of the apoE gene: If there were no apoE genes, mature, filamentous amyloid never formed up to two years of age, compared to massive amyloid by 7 months in the presence of the normal two copies of the apoE gene ([7]; S. Paul, personal communication). One copy of the apoE gene gave intermediate results. In short, human Aβ by itself is incapable of forming amyloid in the mouse without the promoting effect of apoE. A more recent study showed that human apoE driven by the GFAP-promoter could restore filamentous Aβ deposition on an apoE-knockout background, and that human ApoE4 accelerated the amyloid deposition substantially more than did human apoE3 [26]. Evidently, the original *in vitro* experiments that clearly showed apoE to be an amyloid promoter

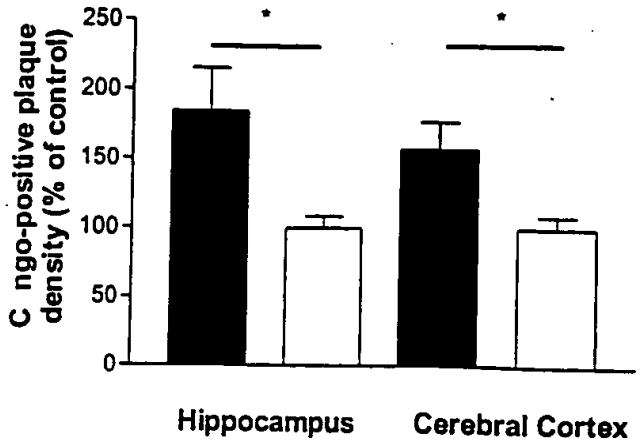

Fig. 2. Transgenic mice carrying both a GFAP-ACT transgene and the PD-APP transgene develop more amyloid deposits by ten months of age than do mice carrying PD-APP alone, demonstrating the amyloid promoting function of ACT *in vivo* (from [54]; copyright 2001, The Society for Neuroscience).

must have come close to mimicking the *in vivo* situation in the Alzheimer brain in that they reflected the increased pathogenic nature of apoE4 in the human disease.

Because transgenic mice have been important in establishing the role of APP and apoE in Alzheimer amyloid formation, we sought to develop transgenic mice expressing human ACT in the brain to test the function of this inflammatory protein *in vivo*. First, we developed a plasmid capable of expressing large amounts of human ACT in mouse astrocytes. Transgenic mice were then generated using the GFAP/ACT expression plasmid and conventional oocyte injection. The ACT transgenic animals are viable with no overt pathological signs and express fully glycosylated ACT protein (~68 kDa) in the brain that comigrates with human plasma ACT protein. The ACT mice have been crossed to the Exemplar/Athena PDAPP mice [21] to evaluate the effect of ACT on amyloid formation. By ten months, the ACT/APP mice have almost twice the amyloid load and plaque density in the hippocampus and cortex as the mice carrying mutant APP alone ([55]; Fig. 2). Furthermore, since the increased amyloid load was largely due to increased plaque density, particularly of smaller plaques, it is likely that ACT either initiates amyloid filament formation or catalyzes some other early amyloid filament process. The finding that ACT is an amyloid promoter *in vivo* has been independently obtained with a slightly different, less active, ACT transgene by Mucke and Abraham and colleagues [48].

Recently, we have examined mice carrying a mutant human APP gene and the human ACT gene, but lacking any apoE gene. In these mice, the amyloid load is four times higher than in mice carrying APP alone, indicating that both the ACT and apoE proteins function independently as amyloid promoters (Nilsson and Potter in preparation). In the absence of both ACT and apoE, amyloid formation is greatly delayed despite the presence of large amounts of Aβ1-42 expressed from the mutant APP gene.

8. Behavioral studies

Finally, we have tested the various lines of transgenic mice in behavioral tasks of memory and cognition, including the radial arm water maze developed by Arendash and Diamond that is very sensitive to amyloid deposits [22,46]. The preliminary results indicate that the apoE and ACT proteins are needed not only for amyloid formation but also for cognitive decline and memory loss in transgenic mouse models of AD (Nilsson, Potter and Arendash in preparation).

9. Summary

The transgenic animal experiments cap a long series of studies indicating that ACT and apoE and the inflammatory processes that produce these proteins contribute importantly and probably essentially to both amyloid formation and cognitive decline in Alzheimer's disease. Together, the results suggest the basic outlines of a potential pathogenic pathway in Alzheimer's disease that begins with small amounts of Aβ peptide oligomers or protofilaments, is amplified by an IL-1-driven inflammatory cascade, and leads to ACT and/or apoE-promoted amyloid filament formation and neurotoxicity (Fig. 3). It seems likely from the data presented here that early-stage AD pathology first initiates the inflammatory cascade by activating microglia, and is then amplified by the amyloid promoting effect of the induced inflammatory molecules. The precise roles of the different forms of the Aβ peptide and the exact temporal order of the different components of the pathway still remain to be determined. In addition, it is not unreasonable to expect that, besides being amyloid promoters, ACT and apo-E may play other roles in Alzheimer's disease that are more closely related to their normal protease inhibitor and cholesterol transport functions (see for example [1,25,80] and other papers in this Special Issue of the Neurobiology of Aging).

10. Implications for therapeutic intervention

In addition to the potential therapeutic benefit of general anti-inflammatory drugs suggested by the epidemiological studies, the biochemical experiments identify potential foci of more specific therapeutic intervention. For example, it may be possible to develop molecules that inhibit the interaction between ACT and Aβ or between apoE and Aβ and prevent the accelerated formation of amyloid filaments. We have already begun to identify such molecules in the form of Aβ-related peptides [42]. The *in vitro* experiments indi-

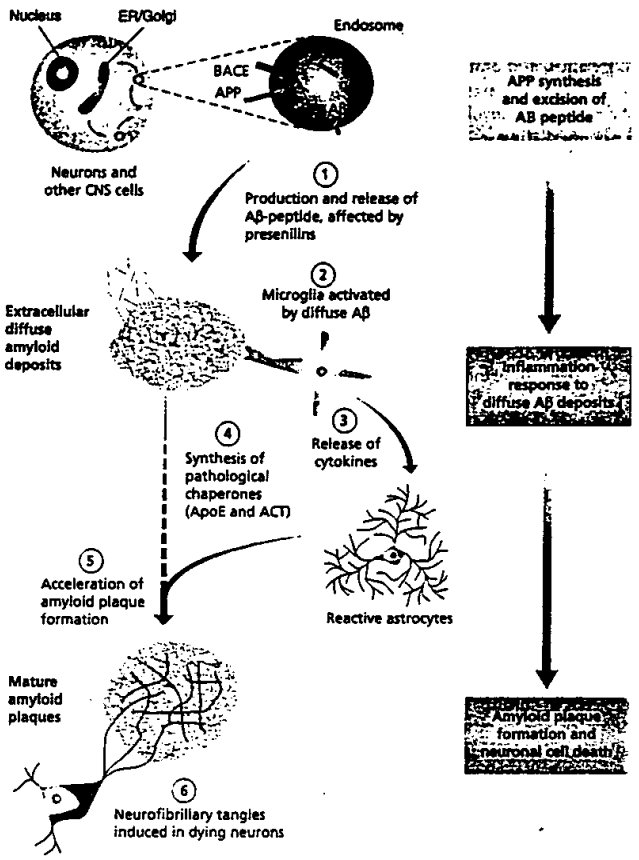

Fig. 3. A diagram of our current understanding of the series of steps (the pathogenic pathway) that leads to Alzheimer's disease, including the essential role of inflammation and its products. (from [61]).

cate that the introduction of these blocking peptides into the brains of the experimental transgenic animals, for instance by expression from adeno-associated virus vectors, should inhibit ACT or apoE-promoted amyloid formation. The first such vector has been constructed and will be used to block apoE binding to Aβ in the APP transgenic mice. The blocking peptides may also serve as the basis for the intelligent design of small molecules with similar decoy properties. Finally, we have found that blocking the IL-1 receptor on astrocytes *in vitro* or treating the cells with anti-inflammatory agents prevents their induced expression of ACT and APP ([13]; Rogers and Potter in preparation). If such blockade of IL-1 function can be accomplished specifically in the brain, it should eliminate the accelerating effect of the inflammatory cascade and effectively reduce amyloid formation and cognitive decline.

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